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Review

Every methyl counts – Epigenetic calculus

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ABSTRACT

Histone modifications play an important role in the formation of an epigenetic memory system that maintains cellular identity. Their complex patterns have been suggested to constitute a histone code, which encodes for specific forms of chromatin. According to the histone code hypothesis these specific patterns are passed on from one cell generation to the next. This enables cells to keep a specific gene expression pattern even in absence of the specific transcription factors that initiated the expression of lineage determining genes. The methylation of specific lysine residues within the histone tails plays a particularly important role in defining the histone modification pattern as mutations of the enzymes that catalyze the formation or the removal of methyl groups have severe effects on cellular physiology. Lysines can get mono-, di- or trimethylated, but the molecular function of the different modification states is still not fully understood. In the following review we will highlight recent data that try to tackle this question and discuss their potential impact for our understanding of the role of histone methylation in epigenetic inheritance.

1. Introduction

Conrad Waddington coined the term "epigenetics" to describe the interaction between genes and their products that bring the phenotype into being [1]. Today, "epigenetics" specifies the study of inheritable changes in gene expression patterns caused by events other than changes in DNA sequence [2]. These epigenetic changes persist during several cell divisions to maintain a specific gene expression pattern that contributes to the cells identity. The identity of the cell alters during cellular differentiation along with epigenetic changes that are essential to guide a cell from a totipotent to a fully differentiated state [3]. A fully differentiated cell with a determined fate possesses its individual epigenetic signature. Each cell type in an organism has its own specific epigenetic signature that is affected by its genotype, developmental and environmental history and in the end leads to the phenotype of the organism [4]. Various cell types such as neurons, muscle and liver cells are all derived from a single precursor and carry the same genomic information. However, they are obviously distinct from each other and fulfil different functions in the organism. Once a cell has acquired a specific identity, it is important to maintain it by stabilizing the cellular gene expression pattern. This memory is achieved by encoding epigenetic information within the genome [5]. A failure in memory resulting in abrogation of proper gene expression could promote diseases by altering differentiation programs or silencing tumor suppressor genes [6]. Key features that regulate cellular memory include DNA methylation, histone variants, chromatin binding proteins, the position within the nucleus, higher order chromatin structures, nuclear RNA and posttranslational histone modifications, which all act on the chromatin template [2].

Chromatin is a highly dynamic structure consisting of DNA and many associated proteins. The basic building block of chromatin is the nucleosome, which consists of two copies of each of the core histones: H2A, H2B, H3 and H4 [7]. Around this histone octamer 147 base pairs of DNA are wrapped in 1.65 superhelical, left handed turns [8,9]. Histones can carry posttranslational modifications (PTMs) such as acetylation, phosphorylation, ubiquitination, ADP-ribosylation and methylation (Fig. 1). These modifications either alone or in combination actively participate in regulating gene expression patterns by permitting the dynamic access to the DNA and its regulatory elements during DNA replication, DNA repair, transcription and chromosome segregation. Most posttranslational modifications reside on the histone amino termini and are recognized by chromatin binding proteins that translate the information into a specific chromatin structure. A large number of histone modifications have been reported and many of them have been shown to play an important role for a specific process with DNA as the template. It has been proposed that the combination of different histone modifications form a "histone code",

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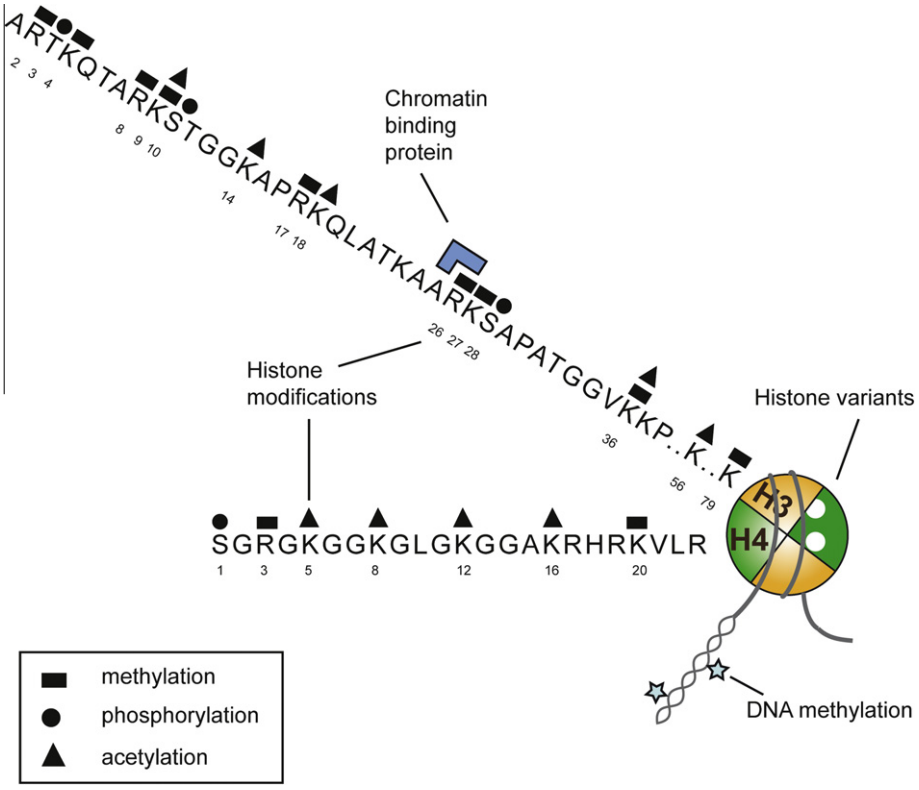

Fig. 1. Epigenetic keyplayer. Chromatin binding proteins, histone variants and different histone modifications are thought to form the "histone code". DNA methylation and posttranslational histone modifications regulate chromatin states.

which specifies patterns of gene expression and eventually results in unique downstream events. This could allow a cell to encode highly complex physiologic processes such as cellular differentiation or the response to certain external signals with a high degree of plasticity and without the need of changing its DNA sequence [10,11]. Due to the analogy to the genetic code that uses the combination of different nucleobases to encode the amino acid sequence of a specific protein, this hypothesis has been very successful in the field of epigenetic research. Schreiber and Bernstein proposed an alternative model for the role of histone modifications in information storage and transfer. They suggest that multiple histone modifications reflect signal switches including positive and negative feedback loops and act similar to signal transduction pathways of receptor tyrosine kinases [12]. In 2004 Henikoff and colleagues suggested that the histone code is not inherited during replication but rather during transcription. Their experiments suggest that the specificity of chromatin states is caused by nucleosomal replacement during transcription with replication independent histone variants [13]. Taken together, the function of histone modifications in regulating epigenetic inheritance still remains to be further investigated.

In this review we would like to focus on the methylation of histones that can either occur on lysines (K) or arginines (R). Currently there are at least 24 sites of lysines and arginines on histones known to be methylated (Fig. 1). On the ε-amino groups of lysines one (me1), two (me2) or three (me3) methyl groups can be added whereas the guanidine nitrogen atoms of argenines can only be mono- or dimethylated [14]. The level of methylation on individual residues allows the generation of complex histone modification patterns within chromatin and the observed stepwise methylation of lysines has the potential to regulate important processes within the nucleus. However, there are many questions we have only recently started to answer: How are the different levels of methylation generated in vivo? Do they have different functions within the cellular context? Is the methylation occurring in a stepwise or processive manner? Is the kinetic of methylation relevant for the function of methylated histones? The molecular function of the different degrees of methylation is currently best studied for the mono-, di- and trimethylation of H4K20. Therefore we would like to primarily discuss this modification as a prime example of what is know about the function of graded methylation and compare it to the different modification at other residues.

2. Enzymology-the writers

Throughout the review we will use the new nomenclature of histone modifying enzymes that has been introduced recently [15]. However, in order to facilitate the understanding of the review for the readers more familiar with the old names we provide a translation table (Table 1).

In the last years several methyltransferases have been identified specific for histone H4K20, which can either be mono-, di- or trimethylated in vivo. The methyltransferase KMT5A specifically monomethylates H4K20 in higher eukaryotes [16,17] whereas di- and trimethylation is mediated by KMT5B and KMT5C, respectively [18,19]. Besides these two activities, the trithorax group activator KMT2H and the nuclear receptor binding SET domain containing protein KMT3B have both been shown to methylate H4K20 peptides [20,21], the functional significance of this activity being still unclear. In contrast to most metazoa, a single enzyme (spKMT5) catalyzes all three methylation states on H4K20 in *Schizosaccharomyces pombe* [22]. In this case the levels of methylation are regulated by the accessory factor Pdp1 that selectively interacts with H4K20me1. A mutation of Pdp1 that impairs its ability to bind H4K20me1 leads to a marked decrease of higher methylation states in vivo [23]. The regulation of methyltransferase activity

Table 1
New nomenclature of histone modifying enzymes. We have used the new nomenclature for histone modifying enzymes [15] throughout the manuscript. The historic names of the human orthologues of histone modifying enzymes used in the manuscript are shown in the left column, the new names in the right column.

Old nomenclature (human)	New nomenclature
Histone methyltransferases	
SUV39H1	KMT1A
MLL1	KMT2A
ASH1	KMT2H
NSD1	KMT3B
DOT1	KMT4
SET9	KMT5
PR-SET7/SET8	KMT5A
SUV4-20H1	KMT5B
SUV4-20H2	KMT5C
EZH2	KMT6
SET7/9	KMT7
Histone demethylases	
LSD1	KDM1
JMJD2A	KDM4A

by associated factors is a common feature of many enzymes. The activity of the H3K4 specific KMT2A enzyme is for example stimulated by the cofactors RbBP5 and WDR5 [24–26]. The exact molecular mechanism by which this stimulation is achieved is controversial but a removal of WDR5 leads to an overall reduction of the trimethylated form of H3K4 [24]. A similar effect of associated factors on trimethylation of particular residues can also be found for the H3K9 specific methyltransferase KMT1E [27] and the H3K27 specific KMT6 containing PRC2 complex [28,29].

Structurally all lysine methylases except KMT4, which specifically methylates K79 on H3, contain a SET domain that harbours their catalytic activity. The SET domain is frequently flanked by a pre-SET and a post-SET domain both of which are, however, not essential for all SET domain-containing enzymes [17]. More structural details have been described by crystallizing several KMTs together with their peptides substrate and the cofactor analog S-adenosylhomocystein (for review see [30]). All SET domain containing methyltransferases have a similar overall structure where the peptide substrate and the cofactor bind to different binding pockets on opposite sides of the protein and are connected via a narrow hydrophobic channel [30]. Structural studies comparing the active sites between the known monomethyltransferases KMT7 or KMT5A with an enzyme that can add up to three methyl groups to its substrates revealed two critical tyrosine residues that coordinate an active site water molecule [31,32]. Enzymes that can add more than one methyl group have a phenylalanine residue at this position, which facilitates that dissociation of the water molecule and enables consecutive rounds of methylation. Consequently, mutation of the Y344 to F in the monomethylase KMT5A leads to a conversion of the enzyme from a mono- to a trimethylase [31].

3. Demethylation-the erasers

Of course the levels of methylation are not only determined by the activity of the enzymes that catalyze the methylation but are also affected by the demethylating enzymes. Like methyltransferases, demethylases are also specific for the different degrees of methylation. The first lysine demethylase described (KDM1) belongs to the class of monoamine oxidases [33] and is only able to remove methyl groups form a mono- or dimethylated lysine (H3K4) as it requires a protonated lysine, which is not present when the lysine is trimethylated [34,35]. The second class of demethylaseses contains a jumonji C domain and is also able to remove methyl groups from trimethylated lysines [35]. The mechanism by which these enzymes remove the methyl groups from lysines is a Fe(II) and 2-oxoglutarate dependent dioxygenation [36]. So far demthylases for several different lysines on H3 (H3K4, 9, 36 and 27) have been reported but only very recently an activity directed against H4K20 was identified [37,38]. In vivo demethylation kinetics of H4K20 in tissue culture cells, showed a very low but higher than zero demethylation rate suggesting that a demethylase with such a specificity exists in HeLa cells at a very low level [39]. It may well be that the demethylases, very much like the methyltransferases, are highly regulated in their activity to ensure the stability of the histone code. KDM1 is for example activated by the association with the CoREST cofactor [40] and can, through association with the androgen receptor, even change its lysine specificity [41]. However, the activity is not only modulated by the composition of KDM enzyme but also by pre-existing modifications on the histone tails, which in turn affect their ability to demethylate certain residues. The demethylase KDM4A for example recognizes the H3 tail when it is methylated at H3K4 or at H4K20 peptides [42] and the demethylase KDM1 is inhibited when the H3 tail is phosphorylated at T6 [43].

4. Function of modifications

Several genome wide profiling studies of different histone modification states have been performed to better understand the function of the different states of methylation [44,45]. In this study, the localization of 36 different histone modifications has been correlated with expression levels of genes that carry the modifications in order to reveal key signatures that are causally related to transcriptional activity in CD4+ T-lymphocytes [45,46]. The analysis of the data revealed that just a single modification had a strong predictive power with a correlation value of 0.72, which was further improved to a value of 0.75 by including an additional two modifications suggesting a high level of redundancy [46]. In this computational analysis the modification that had the highest predictive power was H3K4me3 and H3K79me1 for promoters containing a low content of CpG dinucleotides and H3K27ac and H4K20me1 for promoters with a high content of CpGs [46]. In a less extensive study using developing *Xenopus* embryos, the levels of H3K27me3 have also been show to correlate very well with the repressed state of genes carrying this modification [47] pointing to a critical role of this residue (H3K27) for the regulation of gene activity. However, not all histone modifications may have a role in the regulation of gene activity. In fact several modifications have been shown to occur during chromatin assembly on free histones [48] or shortly after their deposition [49,50]. The acetylation of H3K56 for example has been shown to occur on free histones during their deposition onto DNA [51–53]. Also the monomethylation of H4K20 and the H3K9 are observed during the assembly of chromatin [49,50,54]. The role of monomethylation in chromatin assembly rather than the regulation of gene activity is also supported by the effects of mutations in enzymes that catalyze the modification. Several studies connect the monomethylase activity of KMT5A as an important player in cell cycle progression. During G1 and early S-phase of the cell cycle KMT5A is virtually undetectable due to its rapid CLR4 dependent ubiquitination and degradation, which is dependent on its interaction with PCNA (Proliferating cell nuclear antigen) via a PIP box [55,56]. The interaction is necessary for normal S phase progression [57,58] and the forced expression of KMT5A during S-phase leads to a premature chromatin condensation. In accordance with the KMT5A mRNA and protein levels [59], H4K20me1 increase at late S/G2 and peaks in M phase [60–62] where it is though to mark replication origins for use in the next S-phase [63]. Surprisingly, depletion of KMT5A in

HeLa cells results in no significant changes in cell cycle progression [62] whereas it severely impairs cells S phase progression in U2OS cells suggesting a cell type dependent variability in H4K20me1 function [58,64]. A knock down of KMT5A leads to an increased DNA damage as well as aberrant centrosomes [61]. More striking in KMT5A knockout mice embryonic lethality is observed [60]. Oda and colleagues link DNA damage and cell cycle perturbation to the lack of KMT5A. Moreover, it was recently shown that KMT5A has the potential to methylate p53 leading to speculations whether the association with DNA damage is due to altered p53 effects [65]. In general, H4K20 mono- and trimethylation share low abundance throughout the cell cycle whereas most of H4K20 is dimethylated. Newly synthesized H4 is deposited after the replication fork onto chromatin without methylation marks on K20 in S phase. After chromatin integration H4K20 becomes rapidly monomethylated and then dimethylated during M and G1 phase [62,66].

As described above high resolution profiling of H4K20me1 detected an association of this modification with gene containing regions, where is primarily found at the promoter and coding regions of active genes. This suggests a strong link of H4K20me1 with transcriptional activation [44,67]. A recent study also revealed a link between H4K20 monomethylation and the differentiation of adipocytes. During adipogenesis KMT5A is upregulated tightly coupled to increasing H4K20 monomethylation levels and the knockdown of KMT5A represses adipocyte differentiation [68]. In the system used by Wakabayashi and colleagues, KMT5A expression is upregulated by PPAR gamma during adipogenesis and subsequently acts as a positive regulator of PPAR gamma and its targets thereby generating a positive feedback role that enables a robust induction of adipogenesis. These findings support the finding that H4K20me1 acts as a mark for active chromatin regions and might even be directly recruited to specific promoters to activate genes.

In contrast, a detailed study by Congdon and colleagues mapped several endogenous human genes carrying H4K20me1 on chromosomes 20 and 21 and could detect specific DNA sites that presumably target the H4K20me1 to these sites. Interestingly, in this study H4K20me1 conveys a negative effect on transcription and a knock down experiment leads to a 2-fold up-regulation of the target genes. Reporter assays revealed a transcriptional repression when H4K20me is recruited to a promoter driving a luciferase gene [69]. This repressive effect is consistent with other studies, which

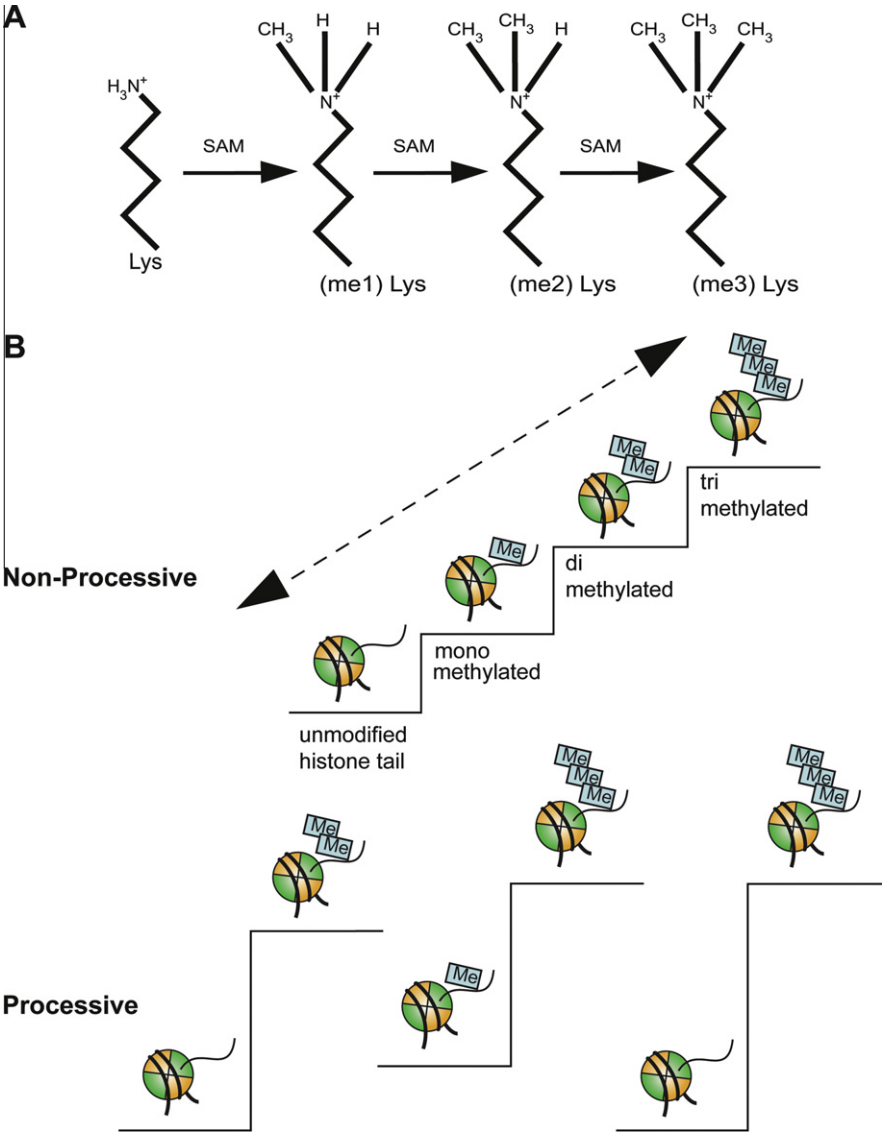

Fig. 2. Stepwise methylation of histone residues. (A) Different methylation states on lysine with S-adenosyl methionin (SAM) as methyl donor. (B) Methylation states can be reached gradually by one step at a time (non-processive enzymes) or by skipping one or two intermediate steps (processive enzymes).

associated monomethylation in undifferentiated ES cells with the inactive X chromosome and also suggest a role in gene silencing [70]. Another link to gene silencing was done in a study investigating *KMT5A* null mutation in *Drosophila melanogaster* [71]. At first glance the two findings correlating H4K20me1 either with active [44,67] or inactive genes [69] are seemingly contradictory and hard to reconcile. However, due to the clear role of H4K20me1 in histone deposition, the correlation with particular gene activity states might simply reflect the degree of histone disruption and reassembly rather than playing an active role in either gene activation or repression.

5. Methylation binding – the readers

Histone modifications are often interpreted by cellular proteins that selectively recognize the modified state of the molecule. Methylation marks recruit a diverse set of proteins to the nucleosome including Tudor domain containing factors classified as the "Royal family" [72]. In case of the methylation of H4K20 several proteins have been shown to bind to the modified residue and even gauge the degree of modification. One of these factors is l(3)MBT that has been shown to bind via a conserved malignant brain tumor motif to H4K20me1 and H1bK26m1/2. In contrast to other domains that selectively recognize di- and trimethylated lysines, l(3)MBT has a preference to lower methylation states [73]. This is due to differences within the conserved aromatic binding pocket of the l(3)MBT binding domain and the domains of known trimethyl lysine binders. In l3(MBT) one of the three aromatic amino acids that bind the trimethylated lysine groups in other Tudor domains has been replaced by a acidic residue thereby facilitating a hydrogen bond between the carboxyl group in the binding pocket binding domain and the methyl ammonium proton of the methylated lysine. Trojer and colleagues showed that the binding of l(3)MBT leads to a compaction of nucleosomal arrays by bridging to modified nucleosomes [74]. This binding then leads to a repressed chromatin structure at sites where l(3)MBT is found within the genome [74]. At the same time H4K20me1 together with l(3)MBT is also recruited newly assembly chromatin where it facilitates the removal of deposition dependent acetylations on histone H4 by virtue of its interaction with the deacetylase Rpd3 [49].

In contrast to the selective recognition of HK20me1 by l(3)MBT, H4K20me3 is able to recruit several DNA repair factors [75]. In *S. pombe* the checkpoint protein Crb2 is associated with sites of DNA damage and requires H4K20 methylation preferably dimethylation. Lack of Crb2 binding to H4K20me leads to double strand breaks [76]. In mammals the Crb2 homolog 53BP1 binds to H4K20 methylation in the context of DNA repair [23,75]. Structural studies indicate a preferential binding to the dimethylation state [75]. Hypothetically as a result of DNA breaks the accessibility of H4K20 methylation is increased in order to facilitate the binding of checkpoint proteins. Like most trimethylated states, trimethylation of H4K20 is of rather low abundance [62,77]. Similar to H3K9me3 it is enriched in pericentromeric heterochromatin [18] but can also be found at mammalian telomeres which is dependent on the trimethylases KMT5B and C [78]. Both enzymes are described to bind to HP1 in vitro suggesting a direct involvement of heterochromatin formation. Moreover members of the retinoblastoma (RB) family are also associated with KMT5B and C methyltransferases. Gonzalo et al. showed a drastic decrease in pericentric and telomeric chromatin of H4K20me3 upon the lack of all 3 RB proteins [79].

6. Conclusion

Clearly the different degrees of lysine methylation are tightly regulated in vivo. This is not only achieved by the existence of specific enzymes that catalyze the different methylation states but also by binding factors that differentiate between the three states of methylation. The functional significance of this tight regulation, however, is still unclear. There seems to be a clear kinetic difference in the formation of the different states of histone methylations with the mono methylations being established more rapidly [66] and having a much higher turnover rate than the higher states of methylations [39]. Although this has been best studied in the case of H4K20 [62] similar kinetics are found for most cases of histone lysine methylation.

Many histone methyltransferases are able to catalyze the formation of all three methylation states. Some of them are processive enzymes that, once bound to a histone tail, lead to the formation of trimethylated histones in vitro (Fig. 2). Others meth-

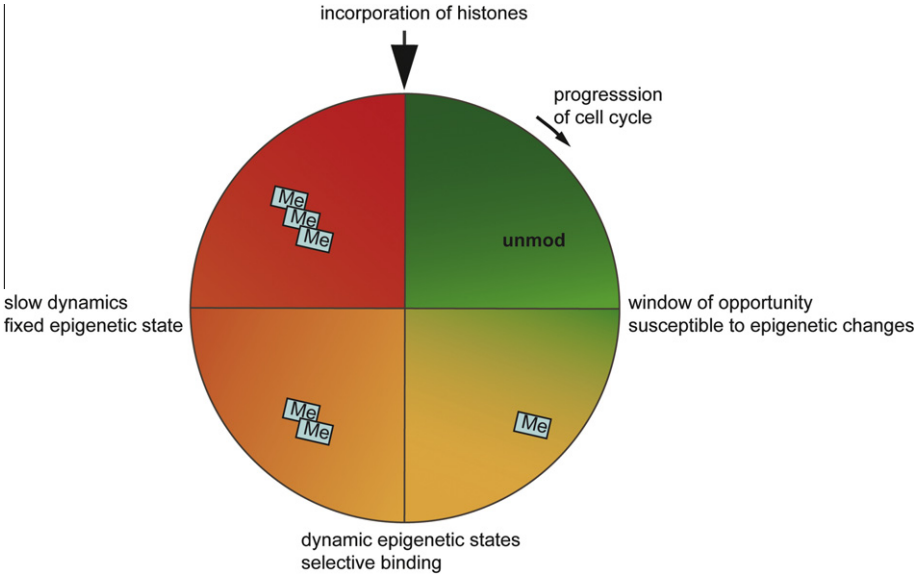

Fig. 3. Slow imprinting of methylation states. Depending on the specific state of histone methylation, the introduction of epigenetic changes may be easier or more challenging. Shortly after histone incoorporation (arrow on top) and thanks to the slow harmonization of histone methylation marks the epigentic fate is transparent for almost a full cell cycle. This fuzzyness of the histone marks generates a window of opportunity during which epigentic changes can take place.

ylate the histone tail in a sequential manner which makes the formation of highly (tri-) methylated histones dependent on the presence of the lower (mono-) methylated state. In both cases, the observed slower formation of higher methylated states in vivo may simply reflect the residence time of the enzyme that modifies it. The known heterochromatic H3K9 mehyltransferase KMT1A for example spends more time within a heterochromatic domain of the eukaryotic nucleus than in euchromatin regions through its interaction with the heterochromatin binding protein HP1 [80]. As HP1 in turn binds to chromatin methylated at H3K9 this interaction forms an autoregulatory loop leading to an increased level of trimethylation. In case of the PRC2 complex the subunit EED binds to H3K27me and is thought to tether the catalytic subunit KMT6 to regions with a high density of this modification [28]. Besides this simple mass action model, the multiple levels of methylation may of course also reflect the concentration and/or the targeting of specific enzymes that specialize on various degrees of methylations such as KMT5A and KMT5B and C in the case of H4K20. Finally the degree of methylation may also be regulated by extrinsic or intrinsic signals that modulate the activity of the enzyme either by availability of the corresponding co-factors or by modifying the enzymes itself.

The question of functional significance, however, remains. Why is it important for a given locus to carry a mono-, di- or trimethylated histone? One possible explanation is the maintenance of epigenetic plasticity. After the assembly of new histones into chromatin during S-phase, the chromatin contains a mosaic pattern of histone methylations. As long as the patterns are not harmonized (new histones adopting the same pattern as the neighboring old ones) the possibility of epigenetic changes is high. This may also explain why actively dividing stem cells with short G1 and G2 phases are more susceptible for a variety of external signals that induce a particular differentiation signal. Fully differentiated cells, however, are either not dividing at all and therefore have a very homogenous modification pattern or divide very slowly to allow sufficient time to harmonize the modification patterns (Fig. 3). It is tempting to speculate that the mis-regulation of histone modifying enzymes in many cancers might generate an increased level of epigenetic instability similar to the disturbing effect many tumorigenic mutations have on genetic stability. Therefore future therapeutic advances have started to take the increased epigenetic instability into account and target the enzymes that deposit or remove histone modifications [6].

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